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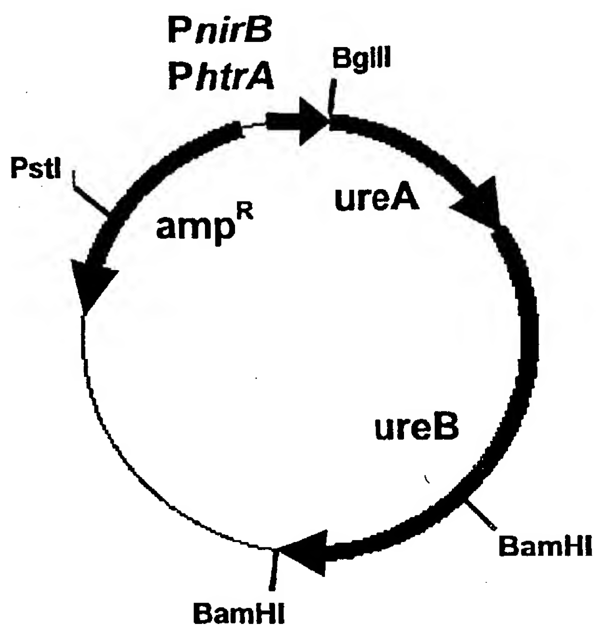
(54) Title: USE OF SALMONELLA VECTORS FOR VACCINATION AGAINST HELLICOBACTER INFECTION



(57) Abstract: The invention provides a method of immunization against Helicobacter, involving mucosal administration of an attenuated Salmonella vector including a nucleic acid molecule encoding a Helicobacter antigen, and parenteral administration of a soluble Helicobacter antigen, co-administered with a suitable parenteral adjuvant. Also provided by the invention are attenuated Salmonella vectors for use in this method.

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USE OF SALMONELLA VECTORS FOR VACCINATION AGAINST HELICOBACTER INFECTION

Background of the Invention

This invention relates to the use of Salmonella vectors in vaccination methods against Helicobacter infection.

Helicobacter is a genus of spiral, gram-negative bacteria that colonize the gastrointestinal tracts of mammals. Several species colonize the stomach, most notably *H. pylori, H. heilmanii, H. felis*, and *H. mustelae*. Although *H. pylori* is the species most commonly associated with human infection, *H. heilmanii* and *H. felis* have also been isolated from humans, but at lower frequencies than *H. pylori*. Helicobacter infects over 50% of adult populations in developed countries and nearly 100% in developing countries and some Pacific rim countries, making it one of the most prevalent infections worldwide.

Helicobacter is routinely recovered from gastric biopsies of humans with histological evidence of gastritis and peptic ulceration. Indeed, *H. pylori* is now recognized as an important pathogen of humans, in that the chronic gastritis it causes is a risk factor for the development of peptic ulcer diseases and gastric carcinoma. It is thus highly desirable to develop safe and effective methods for preventing and treating Helicobacter infection.

Summary of the Invention

The invention provides a method of inducing an immune response against Helicobacter in a mammal. This method involves mucosally (*e.g.*, orally) administering to a mammal (*e.g.*, a human) an attenuated Salmonella (*e.g.*, *S. typhi* (*e.g.*, CVD908-htrA or CVD908) or *S. typhimurium* (*e.g.*,

BRD509 or BRD807)) vector including a nucleic acid molecule encoding a Helicobacter antigen (*e.g.*, a urease, a urease subunit, or an immunogenic fragment thereof), and parenterally administering to the mammal a Helicobacter antigen (*e.g.*, a urease, a urease subunit, or an immunogenic fragment thereof), optionally, in association with an adjuvant, such as an aluminum compound (*e.g.*, alum). The nucleic acid molecule encoding the Helicobacter antigen can be under the control of a promoter, such as an *htrA* or a *nirB* promoter. The antigen used in the mucosal administration can be different from, cross-reactive with, or, preferably, identical to the parenterally administered antigen. In a preferred embodiment, the mucosal administration primes an immune response to an antigen, and the parenteral administration boosts an immune response to the antigen. A mammal treated according to the method of the invention can be at risk of developing, but not have, a Helicobacter infection, or can have a Helicobacter infection. That is, the method can be used to prevent or to treat Helicobacter infection.

The invention also includes use of the Salmonella vectors described above in the preparation of medicaments for preventing or treating Helicobacter (*e.g.*, *Helicobacter pylori*) infection by mucosal (*e.g.*, oral) administration of the vectors, and parenteral administration of a Helicobacter antigen (*e.g.*, a urease, a urease subunit, or an immunogenic fragment thereof; also see other antigens listed herein), optionally in association with an adjuvant (*e.g.*, alum).

The invention also provides an attenuated Salmonella (*e.g.*, *S. typhi* (*e.g.*, CVD908-htrA or CVD908) or *S. typhimurium* (*e.g.*, BRD509 or BRD807)) vector including a nucleic acid molecule encoding a Helicobacter antigen, *e.g.*, a urease, a urease subunit, or an immunogenic fragment thereof, expressed as a fusion protein that can be selectively

targeted to the outer membrane or secreted from the cell. The nucleic acid molecule encoding the Helicobacter antigen can be under the control of a promoter, such as an *htrA* or a *nirB* promoter.

Other features and advantages of the invention will be apparent from the following detailed description, the drawings, and the claims.

Brief Description of the Drawings

Fig. 1 is a schematic representation of an expression plasmid (pH/NUR3) used in Salmonella immunizations.

Fig. 2A is a graph showing the urease-specific serum antibody (IgG2a) response of mice that were mucosally primed with *S. typhimurium*-vectored urease, followed by parenteral boosting with urease and alum.

Fig. 2B is a graph showing the T helper phenotype (IgG1/IgG2a ratio) of mice that were mucosally primed with *S. typhimurium*-vectored urease, followed by parenteral boosting with urease and alum.

Fig. 3A is a graph showing protection against Helicobacter infection in mice that were mucosally primed with *S. typhimurium*-vectored urease, followed by parenteral boosting with urease and alum.

Fig. 3B is a table showing protection against Helicobacter infection in mice that were mucosally primed with *S. typhimurium*-vectored urease, followed by parenteral boosting with urease and alum, as $\log_{10}$ reduction in comparison to a no treatment control group.

Fig. 4 provides the nucleic acid sequence (SEQ ID NOs:1 and 19) and amino acid sequence (SEQ ID NOs:2-18 and 20-30) of plasmid pHUR3.

Fig. 5 is a schematic representation of some relevant features of pHUR3.

Detailed Description

This invention provides an immunization method against Helicobacter infection that involves: (i) mucosal administration of an attenuated Salmonella vector containing a nucleic acid molecule encoding a Helicobacter antigen, and (ii) parenteral administration of a Helicobacter antigen, preferably, in association with an adjuvant. The method can be used to prevent or to treat Helicobacter infection in a mammal, such as a human. Also, the mucosal administration can be used to prime an immune response to an antigen, and the parenteral administration can be used to boost an immune response to the antigen. The invention also provides Salmonella vectors for use in this method. Salmonella vectors, Helicobacter antigens, and adjuvants that can be used in the method of the invention are first described, as follows. Then, details of the immunization method of the invention, and examples of its efficacy, are provided.

Salmonella Vectors

Numerous attenuated Salmonella vectors that can be used in the invention are known in the art, and can be derived from species such as, for example, *S. typhi*, *S. typhimurium*, *S. enteritidis*, *S. dublin*, *S. minnesota*, and *S. choleraesuis*. The vectors can be attenuated chemically (*e.g.*, Ty21a, Swiss Serums and Vaccines, Berna Products) or, preferably, by genetic mutagenesis (*e.g.*, Ty800). For example, attenuation can be achieved by inactivation of key regulatory genes or genes necessary for *in vivo* survival. For example, the following genes can be inactivated: *cya*, *crp*, and *asd* (cAMP metabolism; see, *e.g.*, Curtiss *et al.*, Vaccine 6:155-160, 1988; Nakayama *et al.*, BioTechnology 6:693, 1988; WO 92/11361), adenylate cyclase and the cAMP receptor (U.S. Patent No. 5,389,368), *cdt* (invasion of liver and spleen), *phoP/phoQ* (two component regulator; see, *e.g.*, Fields

et al., Science 243:1059-1062, 1989; U.S. Patent No. 5,424,065), *ompR* (control of capsule and porin expression; see, *e.g.*, Dorman *et al.*, Infection and Immunity 57:2136-2140, 1989), outer membrane proteins (U.S. Patent No. 5,527,529), reverse mutants of streptomycin mutants (U.S. Patent No. 4,350,684), genes in pathogenicity islands (Shea *et al.*, Infection and Immunity 67:213-219, 1999; WO 99/37759), *SPI-2* (invasion of Peyer's patches), *Dam* (DNA methylation), *htrA* (heat shock protein; U.S. Patent No. 5,804,194), and other heat shock proteins (U.S. Patent No. 5,804,194). The vectors can also be attenuated by auxotrophic mutations, such as mutations in any of the *aroA*, *aroC*, *aroD* (aromatic compounds), *purA*, or *guaAB* (purines) genes (see, *e.g.*, U.S. Patent No. 5,770,214).

Preferably, the mutations in the Salmonella strains used in the invention are non-reverting mutations, *i.e.*, mutations that cannot be repaired in a single step. Mutations of this sort include deletions, inversions, insertions, and substitutions. Preferably, there is more than one mutation in the vector. Methods of making such mutations are well known in the art.

Specific examples of Salmonella vectors that can be used in the invention include *S. typhi* mutant strains, for example, CVD908 *S. typhi* Ty2 ΔaroC/ΔaroD (Hone *et al.*, Vaccine 9:810-816, 1991), CVD908-htrA *S. typhi* Ty2 ΔaroC/ΔaroD/ΔhtrA (Tacket *et al.*, Infection and Immunity 65:452-456, 1997), BRD1116 *S. typhi* Ty2 ΔaroA/ΔaroC/ΔhtrA (Lowe *et al.*, Infection and Immunity 67:700-707, 1999), *S. typhi* ΔaroA/ΔaroE (U.S. Patent No. 5,770,214; deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No. NCTC 12164), *S. typhi* Ty2 ΔaroA/ΔaroC Km-R (U.S. Patent No. 5,770,214; deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No. NCTC 12165), and *S. typhi* ΔaroA/ΔaroD (U.S. Patent No. 5,770,214;

deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No. NCTC 122309). It has been shown that one of these, CVD908-htrA, is safe and immunogenic in phase I (Tacket *et al.*, Infection and Immunity 65:452-456, 1997) and phase II studies in a total of 100 adult volunteers.

Specific examples of *S. typhimurium* mutant strains that can be used in the invention include BRD509 *S. typhimurium* ΔaroA/ΔaroD (Strugnell *et al.*, Infection and Immunity 60:3994-4002, 1992), BRD807 *S. typhimurium* ΔaroA/ΔhtrA (Chatfield *et al.*, Microbial Pathogenesis 12:145-151, 1992; U.S. Patent No. 5,804,194; deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No. NCTC 12459), BRD698 (U.S. Patent No. 5,804,194; deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No. NCTC 12457), and BRD726 (U.S. Patent No. 5,804,194; deposited at PHLS, NCTC, 61 Colindale Avenue, London NW9 5HT under Accession No. NCTC 12458).

Additional examples of Salmonella mutant strains that can be used in the invention are described in the following publications: double *aro* mutants (WO 89/05856, U.S. Patent No. 5,770,214), *htrA* mutants (WO 91/15572, U.S. Patent No. 5,804,194), and *ompR* mutants (U.S. Patent No. 5,527,529). Also see, for example, Nakayama *et al.*, BioTechnology 6:693, 1988 and WO 92/11361. In addition, there are numerous alternative strains of *S. typhi* and *S. typhimurium* described in the literature or known in the art that are also attenuated in their virulence, and have been shown to induce immune responses against heterologous antigens. Any of these strains can be used in the method of the present invention.

Any of the attenuated Salmonella strains described above, or others, can be used in the method of the invention to administer a Helicobacter antigen to a mammal for vaccination against Helicobacter infection. This can be accomplished by introducing into the attenuated Salmonella strain a nucleic molecule encoding a Helicobacter antigen. The antigen-encoding nucleic acid molecule to be introduced into the attenuated Salmonella strain can be present, for example, in a plasmid vector (*e.g.*, pHUR3, pHUR4, pNUR3, or pNUR4 (see below)) that includes a regulatory sequence, such as a promoter, and, optionally, a sequence encoding a secretion signal (*e.g.*, a bacterial hemolysin (hly) secretion signal; WO 87/06953, U.S. Patent No. 5,143,830).

The promoter can be a prokaryotic promoter, for example, a Salmonella promoter, which directs expression of the Helicobacter antigen in the Salmonella vector. Examples of such promoters include the *htrA* promoter (WO 95/20665), the *nirB* promoter (WO 92/15689, U.S. Patent No. 5,547,664), the *ssaH* promoter (Valdivia *et al.*, Science 277:2007-2011, 1997), the *ompR* promoter, and any other Salmonella or other bacterial promoter that is upregulated when Salmonella is taken up by mammalian cells. Alternatively, the promoter can be a eukaryotic promoter, such as the cytomegalovirus promoter. Use of such promoters allows for expression of target antigen in a eukaryotic cell, with Salmonella acting as the delivery vehicle for this DNA immunization approach. The construction of such vectors is known in the art. Of course, numerous eukaryotic promoters are known in the art and can be used in the invention.

Introduction of a plasmid into an attenuated Salmonella strain can be accomplished using any of a number of standard methods, such as electroporation or bacteriophage transduction (Turner *et al.*, Infection and Immunity 61:5374-5380, 1993). Also see, *e.g.*, Ausubel *et al.*, *Current*

Protocols in Molecular Biology, John Wiley & Sons Inc., 1994, and Ward *et al.*, Infection and Immunity 67(5):2145-2152, 1999, for methods of introducing plasmids into bacteria, such as Salmonella.

Helicobacter Antigens

Preferred antigens for use in the invention are Helicobacter (*e.g.*, *H. pylori* or *H. felis*) proteins (*i.e.*, peptides or polypeptides), other components Helicobacter (*e.g.*, lipopolysaccharides, carbohydrates, or nucleic acid molecules), or immunogenic fragments thereof. Preferably, the same or a similar (*e.g.*, a fragment) antigen is used in the mucosal administration step as in the parenteral administration step, however, the antigen used in each of these steps can differ. Also, preferably, the mucosally administered antigen primes an immune response to the antigen, and the parenterally administered antigen boosts an immune response to the same antigen. For the mucosal administration step, a nucleic acid molecule (*e.g.*, a DNA molecule) encoding a desired antigen is inserted into an attenuated Salmonella vector, as is described above. For the parenteral administration step, the antigen can be, for example, purified from a bacterial culture or produced using standard recombinant or chemical synthetic methods. Methods for identifying immunogenic fragments of polypeptide antigens are known in the art, and can be employed in preparing antigens for use in the method of the invention (see, *e.g.*, Sturniolo *et al.*, Nature Biotechnology, "Generation of Tissue-Specific and Promiscuous HLA Ligand Databases Using DNA Microarrays and Virtual HLA Class II Matrices," June, 1999). Additional antigens that can be used in the parenteral administration step are whole Helicobacter bacteria and non-purified protein preparations, such as Helicobacter lysates.

The antigens used in the invention can be produced as fusion proteins, which are polypeptides containing amino acid sequences corresponding to two or more proteins (or fragments thereof) that are normally separate proteins, linked together by a peptide bond(s). Fusion proteins generally are synthesized by expression of a hybrid gene, containing nucleotides encoding each of the individual polypeptides that make up the fusion protein. An example of an antigenic fusion protein that can be used in the invention is one that contains a cholera toxin (CT) or an *E. coli* heat-labile toxin (LT) adjuvant (*e.g.*, a toxin A or B subunit, or a fragment or derivative thereof having adjuvant activity) fused to an *H. pylori* antigen, *e.g.*, a urease antigen. Another type of fusion protein included in the invention consists of an antigen fused to a polypeptide (*e.g.*, glutathione S-transferase (GST)) that facilitates purification of the fusion protein. Still another type of fusion protein that can be used in the invention is a fusion with a polypeptide that targets the expressed protein to cells of the immune system. For example, fusions with CD4 or Staph A can be used. Proteins used as antigens in the invention can also be covalently coupled or chemically cross-linked to adjuvants, using standard methods.

The most preferred *H. pylori* antigens for use in the invention are urease antigens, which include, *e.g.*, immunogenic fragments or subunits (*e.g.*, UreA or UreB) of urease. Most preferred urease antigens are enzymatically inactive, recombinant multimeric urease complexes, produced as described in Lee *et al.*, WO 96/33732. A number of other immunogenic *H. pylori* antigens can be administered according to the invention, *e.g.*, catalase (WO 95/27506), HspA and HspB (WO 94/26901), lactoferrin receptor (WO 97/13784), p76 (WO 97/12908), p32 (WO 97/12909), BabA and BabB (WO 97/47646), AlpA (WO 96/41880), AlpB (WO 97/11182), as well as the antigens described in WO 96/38475, WO

96/40893, WO 97/19098, WO 97/37044, WO 98/18323, WO 97/37044, WO 97/4764, WO 98/04702, and WO 98/32768. Additional preferred antigens for use in the invention are GHPO 1516, GHPO 789, GHPO 386, GHPO 1615, GHPO 1360, GHPO 1320, GHPO 639, GHPO 792, GHPO 536, GHPO 525, GHPO 1275, GHPO 1688, GHPO 706, GHPO 419, GHPO 1595, GHPO 1398, GHPO 986, GHPO 1282, GHPO 1056, GHPO 1443, GHPO 13, GHPO 109, GHPO 257, GHPO 1034, GHPO 236, GHPO 1166, GHPO 1351, and GHPO 1420 (WO 98/21225, WO 98/43478, and WO 98/43479), as well as other antigens described in these publications.

Adjuvants

Although not required, the attenuated Salmonella vectors described above for mucosal administration step can be administered with a mucosal adjuvant. The adjuvant can be admixed with the Salmonella vector or expressed in the Salmonella vector (*e.g.*, as a fusion protein with an antigen, see above), either from an integrated nucleic acid molecule or episomally, *e.g.*, on a plasmid. Such adjuvants can be chosen from bacterial toxins, *e.g.*, the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin, and the Pertussis toxin (PT), or combinations, subunits, toxoids, fragments, homologs, derivatives, fusions, or mutants that are derived therefrom and have adjuvant activity. For example, it is possible to use a purified preparation of the native cholera toxin B subunit (CTB) or a polypeptide including the carboxyl-terminal repeats of *C. difficile* toxin A (WO 97/02836). Preferably, a mutant is used in which toxicity is reduced. Such mutants are described in, *e.g.*, WO 95/17211 (mutant CT Arg-7-Lys), WO 96/6627 (mutant LT Arg-192-Gly), and WO 95/34323 (mutant PT Arg-9-Lys and Glu-129-Gly). Other LT mutants that can be used include at least one of the following mutations: Ser-63-Lys, Ala-69-Gly,

Glu-110-Asp, and Glu-112-Asp. Other compounds, such as MPLA, PLGA, and QS-21, can also be used as adjuvants for the mucosal route.

Adjuvants for use in parenteral administration include, for example, aluminum compounds (*e.g.*, alum), such as aluminum hydroxide, aluminum phosphate, and aluminum hydroxy phosphate. The antigen can be precipitated with, or adsorbed onto, the aluminum compound using standard methods.

In addition to aluminum compounds, a large number of appropriate adjuvants for administration by the systemic or parenteral route exist in the art and can be used in the invention. For example, liposomes; ISCOMS; microspheres; protein chochleates; vesicles consisting of nonionic surfactants; cationic amphiphilic dispersions in water; oil/water emulsions; muramidyldipeptide (MDP) and its derivatives, such as glucosyl muramidyldipeptide (GMDP), threonyl-MDP, murametide, and murapalmitin; QuilA and its subfractions; as well as various other compounds, such as DC-chol; monophosphoryl-lipid A (MPLA) major lipopolysaccharide from the wall of a bacterium, for example, *E. coli*, *S. minnesota, S. typhimurium*, *Shigella flexneri*, or *N. meningitidus*; algan-glucan; gamma-inulin; calcitriol; and loxoribine can be used. Other adjuvants, such as RIBI (ImmunoChem, Hamilton, MT) and polyphosphazene (WO 95/2415), can also be used in parenteral administration.

Useful liposomes for the purposes of the present invention can be selected, for example, from pH-sensitive liposomes, such as those formed by mixing cholesterol hemisuccinate (CHEMS) and dioleyl phosphatidyl ethanolamine (DOPE); liposomes containing cationic lipids recognized for their fusiogenic properties, such as 3-beta-(N-(N',N'-dimethylamino-ethane)carbamoyl)cholesterol (DC-chol) and its equivalents, which are

described in U.S. Patent No. 5,283,185 and WO 96/14831; dimethyldioctadecylammonium bromide (DDAB) and the BAY compounds described in EP 91645 and EP 206 037, for example, Bay R1005 (N-(2-deoxy-2-L-leucylamino-beta-D-glucopyranosyl)-N-octa-decyldodecanoylamide acetate; and liposomes containing MTP-PE, a lipophilic derivative of MDP (muramidyldipeptide). These liposomes are useful as adjuvants with all of the antigens described herein.

Useful ISCOMs for the purposes of the present invention can be selected, for example, from those compounds of QuilA or of QS-21 combined with cholesterol and, optionally, also with a phospholipid, such as phosphatidylcholine. These are particularly advantageous for the formulation of the lipid-containing antigens.

Useful microspheres for the purposes of the present invention can be formed, for example, from compounds such as polylactide-co-glycolide (PLAGA), alginate, chitosan, polyphosphazene, and numerous other polymers.

Useful protein chochleates for the purposes of the present invention can be selected, for example, from those formed from cholesterol and, optionally, an additional phospholipid, such as phosphatidylcholine. These are especially advantageous for the formulation of the lipid-containing antigens.

Useful vesicles consisting of nonionic surfactants for the purposes of the present invention can be, for example, formed by a mixture of 1-mono-palmitoyl glycerol, cholesterol, and dicetylphosphate. They are an alternative to conventional liposomes, and can be used for the formulation of all of the antigens described herein.

Useful oil/water emulsions for the purposes of the present invention can be selected, for example, from MF59 (Biocine-Chiron), SAF1 (Syntex), and the montanides ISA51 and ISA720 (Seppic).

A useful adjuvant for the purposes of the present invention can also be a fraction derived from the bark of the South American tree *Quillaja Saponaria Molina*, for example, QS-21, a fraction purified by HPLC chromatography as is described in U.S. Patent No. 5,057,540. Since some toxicity may be associated with QS-21, it may be advantageous to use it in liposomes based on sterol, as is described in WO 96/33739.

Induction of an Immune Response Against Helicobacter

The method of the invention can be used to prevent Helicobacter infection in a patient, as well as to treat an ongoing Helicobacter infection in a patient. Thus, gastroduodenal diseases associated with these infections, including acute, chronic, or atrophic gastritis, and peptic ulcers, *e.g.*, gastric or duodenal ulcers, can be prevented or treated using the method of the invention.

As is noted above, the method of the invention involves mucosal (*e.g.*, oral, intranasal, intragastric, pulmonary, intestinal, rectal, ocular, vaginal, or urinary tract) administration of a Salmonella vector including a nucleic acid molecule that encodes a Helicobacter antigen, followed by parenteral (*e.g.*, intramuscular, subcutaneous, intradermal, intraepidermal, intravenous, or intraperitoneal) administration of a Helicobacter antigen, preferably in association with an adjuvant. The antigen used in the mucosal prime can be different from, cross-reactive with, or, preferably, identical to the parenterally administered antigen. Preferably, the mucosal administration step primes an immune response to an antigen, and the parenteral administration step boosts an immune response to the antigen.

Also included in the invention are vaccination methods involving parenteral priming and mucosal boosting (*e.g.*, with a Salmonella vector including a nucleic acid molecule encoding a Helicobacter antigen), and parenteral administration of a Salmonella vector including a nucleic acid molecule encoding a Helicobacter antigen.

Attenuated Salmonella vectors, antigens, formulations, adjuvants, administration regimens, specific mucosal and parenteral routes, and dosages to be used in the method of the invention can readily be determined by one skilled in the art. For example, 5×10^6 - 5×10^{10} colony forming units, *e.g.*, 5×10^8 colony forming units, of an attenuated Salmonella vector can be used in the mucosal administration, and 5-1000 μg, *e.g.*, 100 μg, antigen, can be used in the parenteral administration. The mucosal administration can take place only once or two or more (*e.g.*, three, four, or five) times, for example, separated by two, three, or four days or weeks. Similarly, the parenteral administration can take place once or two or more (*e.g.*, three, four, or five) times, separated by weeks, months, or years from each other or the mucosal administration.

In one example of an immunization regimen that can be used, a patient is primed with two doses of an attenuated Salmonella vector (*e.g.*, *S. typhi* CVD908-htrA or CVD908, or *S. typhimurium* BRD509 or BRD807) expressing an antigen (*e.g.*, urease from plasmid pHUR3, pHUR4, pNUR3, or pNUR4) on days 0 and 21, and then parenterally boosted on day 51 or later with an antigen (*e.g.*, urease) and an adjuvant (*e.g.*, alum). The details of construction of pHUR3 and pNUR3, which each include an ampicillin resistance gene, are described below. pHUR4 and pNUR4 are constructed by removing the ampicillin resistance gene from pHUR3 and pNUR3, respectively, by digestion with the restriction endonuclease *RcaI*, and

cloning into the digested vectors a kanamycin resistance gene that can be obtained from plasmid pUC4K (Pharmacia) by digestion with *EcoRI*.

A useful pharmaceutical composition for the purposes of the present invention can be manufactured in a conventional manner. In particular, it can be formulated with a pharmaceutically acceptable carrier or diluent, *e.g.*, water or a saline solution. In general, the diluent or carrier can be selected according to the mode and route of administration and according to standard pharmaceutical practices. Appropriate carriers or diluents, as well as what is essential for the preparation of a pharmaceutical composition, are described, *e.g.*, in *Remington's Pharmaceutical Sciences* (18^{th} edition), ed. A. Gennaro, 1990, Mack Publishing Company, Easton, PA., a standard reference book in this field. As a specific example, the attenuated Salmonella vectors of the invention can be formulated in a tablet for oral administration (see, *e.g.*, U.S. Patent No. 5,804,194).

The therapeutic or prophylactic efficacy of the method of the invention can be evaluated according to standard methods, *e.g.*, by measuring the induction of an immune response or the induction of therapeutic or protective immunity using, *e.g.*, the mouse/*H. felis* model and the procedures described in Lee *et al.*, Eur. J. Gastroenterology and Hepatology 7:303, 1995 or Lee *et al.*, J. Infect. Dis. 172:161, 1995. Persons skilled in this art will realize that *H. felis* can be replaced in the mouse model by another Helicobacter species. For example, the efficacy of the method is, preferably, evaluated in a mouse model using an *H. pylori* strain adapted to mice. The efficacy can be determined by comparing the level of infection in gastric tissue (*e.g.*, by measuring the urease activity, bacterial load, or condition of the gastritis) with that in a control group. A therapeutic effect or a protective effect exists when infection is reduced

compared with a control group. Experimental methods and results showing the efficacy of the present method is described as follows.

Experimental methods and results

Construction of ureAB expression plasmids under the control of the nirB and htrA promoters - Method 1

A *ureAB* expression plasmid is constructed by subcloning a PCR product containing the *ureAB* genes (amplified from plasmid pORV273) into plasmid vector ptetnir15. Plasmid pORV273 is obtained from OraVax, Inc., Cambridge, MA. Plasmid ptetnir15 has been described (Chatfield *et al.*, Bio/Technology 10:888-892, 1992; Oxer *et al.*, Nucl. Acids Res. 19:1889-1892, 1991). This vector was modified by standard techniques known in the art, to introduce into the vector a suitable restriction site for subcloning other genes for optimal expression under control of the *nirB* promoter. An *Nco*I site was introduced 10 basepairs 3' to the Shine-Dalgarno sequence of ptetnir15, and the resultant plasmid is designated ptetnir15/mod. Plasmid ptetnir15/mod, carried in strain BRD940, is obtained from Peptide Therapeutics Ltd., Cambridge, U.K.

The *ureAB* gene is amplified by PCR from pORV273 using Turbo Pfu polymerase (Stratagene), which has 3'-5' proof-reading activity, and two primers, designated orafor and orarev. Primer orafor introduces *Eco*RI and *Bsp*HI sites immediately upstream of the initiating codon of the *ureA* gene. Primer orarev binds approximately 18 basepairs downstream of the *Bam*HI site that is located 45 basepairs downstream of the termination codon of the *ureB* gene.

The PCR reaction includes 0.1 μg pORV273 and 100 pmol each of primers orafor (5'-TAG GGA ATT CTC ATG AAA CTC ACC CCA AAA G-3' (SEQ ID NO:31)) and orarev (5'-GCC AAC TTA GCT TCC TTT

CGG G-3' (SEQ ID NO:32)) per 100 µl reaction and utilizes 25 cycles, with an annealing temperature of 50°C. The resulting 2.4 kb PCR product is purified from a 1% agarose gel using a Qiaquick gel extraction kit (Qiagen). As is described below, the actual method used in the generation of pNUR and pHUR differed from this description in the sequence of orarev. Therefore, the method described here may need to be adapted in ways known to those skilled in the art by changing, for example, the precise annealing temperature or the number of cycles required to give sufficient product, or even in the sequence of the primer orarev.

The PCR product is digested with *Bsp*HI + *Bam*HI, and purified with a Promega Wizard DNA clean-up kit. Plasmid ptetnir15/mod is digested with *Nco*I + *Bam*HI (the *Nco*I site is 10 basepairs 3' to the Shine-Dalgarno sequence of ptetnir15, and generates a cohesive end that is compatible with *Bsp*HI), and dephosphorylated using shrimp alkaline phosphatase. The largest fragment from the digestion of ptetnir15/mod is isolated from a 1% agarose gel using a Qiaquick gel extraction kit (Qiagen), and ligated to the digested PCR product using the Ligator Express Kit (Clontech). Ligations are transformed into electrocompetent *E. coli* TG1 cells (Stratagene).

Plasmids from ampicillin-resistant transformants are screened for the presence of the full length, 2.4 kb *ureAB* gene by restriction analysis. The *ureAB* gene from plasmid pORV273 has a *Bam*HI site within the coding sequence. However, in a small number of ptetnir15/mod + *ureAB* transformants, incomplete digestion or re-ligation of the two *ureAB* fragments yields the full length *ureAB* PCR product. The orientation of the *ureAB* gene in the ptetnir15-derived plasmid is confirmed by PCR, and a plasmid with the full length *ureAB* gene, in the correct orientation is designated pNUR.

The *nirB* promoter in plasmid pNUR is replaced with the *htrA* promoter from phtrAcore, which is obtained from Peptide Therapeutics Ltd., Cambridge, U.K. Plasmids pNUR and phtrAcore are digested with *Pst*I and *Bgl*II. Digested pNUR is dephosphorylated with shrimp alkaline phosphatase. The digestion products are run on a 1% agarose gel, and a 0.8 kb fragment containing the *htrA* promoter from the phtrAcore digestion and the 4.0 kb fragment from pNUR lacking the *nirB* promoter are extracted from the gel using a Qiagen Qiaquick gel extraction kit. The two fragments are ligated together (Clontech Ligator express kit), and transformed into electrocompetent *E. coli* TG1 cells (Stratagene). Transformants are screened for the presence of the *htrA* promoter by PCR using primer pairs specific for *htrA* (5902/5904) or *nirB* (5901/5904). A plasmid with the *htrA* promoter and a full length *ureAB* gene is designated pHUR.

The nucleotide sequence across the promoter region and *ureAB* genes of final plasmids are confirmed. Samples of the plasmids are prepared using the Qiagen "Plasmid midi kit" (Catalog No. 12143), and the DNA sequence determined by standard techniques. Oligonucleotides 5901 to 5919 (see below) can be used, and allow nucleotide sequence determination of both DNA strands. Oligonucleotides 5901 and 5902 hybridize within *nirB* and *htrA*, respectively, while 5919 hybridizes within ptetnir15/mod, downstream of the *ureAB* genes. The other oligonucleotides hybridize within the *ureAB* genes. The data confirm that the nucleotide sequence across the recombinant region of all plasmids are as expected.

Plasmids pNUR and pHUR are introduced into *S. typhimurium* strains such as, *e.g.*, BRD509 and BRD807, and *S. typhi* strains such as, *e.g.*, CVD908 and BRD948, by electroporation and selection of ampicillin-resistant colonies.

Construction of ureAB expression plasmids under the control of the nirB and htrA promoters - Method 2

The protocol described above is one example of many by which one skilled in the art can derive an expression plasmid suitable for directing the synthesis of an *H. pylori* antigen, *e.g.*, urease, under the control of the *htrA* or *nirB* promoter in an attenuated strain of Salmonella. Alternative primers can be used in the PCR amplification of the genes from the starting plasmid, and alternative strategies for the introduction of the gene *via* alternative restriction sites are possible. One such alternative was employed in the construction of plasmids pNUR3 and pHUR3. During the design of the primers for PCR, a sequence error in the database-deposited gene sequence caused the 3' end of the *ureB* gene to be incorrectly identified. A primer was synthesized for the PCR amplification that, in fact, resulted in a non-native sequence of the gene, containing an additional 49 codons after the genuine termination codon. This error was subsequently corrected by the method described below, yielding a final plasmid with a sequence identical to that of the plasmid that would be produced by the strategy described above. This method is described in further detail, as follows.

As is described above, plasmid pORV273 was obtained from OraVax Inc. Plasmid ptetnir15 has been described (Chatfield *et al.*, Bio/Technology 10:888-892, 1992; Oxer *et al.*, Nucl. Acids Res. 19:1889-1892, 1991), and this vector was modified by standard techniques, to introduce into the vector a suitable restriction site for subcloning other genes for optimal expression under control of the *nirB* promoter. An *Nco*I site was introduced 10 basepairs 3' to the Shine-Dalgarno sequence of ptetnir15, and the resultant plasmid was designated ptetnir15/mod. Plasmid ptetnir15/mod, carried in strain BRD940, was obtained from the culture collection of Peptide Therapeutics Ltd., Cambridge, U.K.

The ureAB gene was amplified by PCR from pORV273 using Turbo Pfu polymerase (Stratagene), which has 3'-5' proof-reading activity and two primers, designated orafor and orarev. Primer orafor introduces *Eco*RI and *Bsp*HI sites immediately upstream of the initiating codon of the *ureA* gene. Primer orarev introduces a *Bam*HI and a *Pst*I site just before the correct 3' end of the *ureAB* gene. Subsequent digestion and cloning, as is described below, resulted in the deletion of the correct termination codon of *ureB*, with the result that transcription continued into the vector sequence until an in-frame stop codon was reached, adding 49 amino acids to the translated protein.

The PCR reaction included 0.1 μg pORV273 and 100 pmol each of primers orafor (5'-TAG GGA ATT CTC ATG AAA CTC ACC CCA AAA G-3' (SEQ ID NO:31)) and orarev (5'-TCT ACT GCA GGA TCC AAA ATG CTA AAG AGT TGC G-3' (SEQ ID NO:33)) per 100 μl reaction, and utilized 25 cycles, with an annealing temperature of 50°C. The resulting 2.4 kb PCR product was purified from a 1% agarose gel using a Qiaquick gel extraction kit (Qiagen). The PCR product was digested with *Bsp*HI + *Bam*HI, and purified with a Promega Wizard DNA clean-up kit. Plasmid ptetnir15/mod was digested with *Nco*I + *Bam*HI (the *Nco*I site is 10 basepairs 3' to the Shine-Dalgarno sequence of ptetnir15, and generates a cohesive end that is compatible with *Bsp*HI), and dephosphorylated using shrimp alkaline phosphatase. The largest fragment from the digestion of ptetnir15/mod was isolated from a 1% agarose gel using a Qiaquick gel extraction kit (Qiagen), and ligated to the digested PCR product using the Ligator Express Kit (Clontech). Ligations were transformed into electrocompetent *E. coli* TG1 cells (Stratagene).

Plasmids from ampicillin-resistant transformants were screened for the presence of the full length, 2.4 kb *ureAB* gene by restriction analysis. The *ureAB* gene from plasmid pORV273 has a *Bam*HI site within the coding sequence. However, in a small number of ptetnir15/mod + *ureAB* transformants, incomplete digestion or re-ligation of the two *ureAB* fragments yielded the full length *ureAB* PCR product. The orientation of the *ureAB* gene in the ptetnir15-derived plasmid was confirmed by PCR and a plasmid with the full length *ureAB* gene, in the correct orientation was designated pNUR1.

The *nirB* promoter in plasmid pNUR1 was replaced with the *htrA* promoter from phtrAcore, which is obtained from Peptide Therapeutics Ltd., Cambridge, U.K. Plasmids pNUR1 and phtrAcore were digested with *Pst*I and *Bgl*II. Digested pNUR1 was dephosphorylated with shrimp alkaline phosphatase. The digests were run on a 1% agarose gel, and a 0.8 kb fragment containing the *htrA* promoter from the phtrAcore digest and the 4.0 kb fragment from pNUR1 lacking the *nirB* promoter were extracted from the gel using a Qiagen Qiaquick gel extraction kit. The two fragments were ligated together (Clontech Ligator express kit) and transformed into electrocompetent *E. coli* TG1 cells (Stratagene). Transformants were screened for the presence of the *htrA* promoter by PCR using primer pairs specific for *htrA* (5902/5904) or *nirB* (5901/5904). A plasmid with the *htrA* promoter and a full length *ureAB* gene was designated pHUR1.

Subsequent to this it was discovered that there had been a cloning error in the 3' terminal portion of *ureB*, resulting in a translated product with an additional 49 amino acids from both pHUR1 and pNUR1. This was corrected by replacing the small *Bam*HI fragment containing the C-terminus of the *ureB* gene with the corresponding, and correct, fragment from pORV272. pORV273, pHUR1, and pNUR1 were digested with *Bam*HI,

and the small fragment from the pORV273 digestion was ligated to the large fragment from the pHUR1 and pNUR1 digestions. Clones were screened for orientation of the insert, and clones with the correct orientation were designated pHUR3 and pNUR3. These clones were characterized by full nucleotide sequencing of the region including the promoter and the complete *ureAB* gene on both strands, and found to be correct.

The nucleotide sequences across the *nirB* promoter and *ureAB* genes of pNUR1 and of the *htrA* promoter region of pHUR1 were confirmed. Samples of the two plasmids were prepared using the Qiagen "Plasmid midi kit" (Catalogue No. 12143), and the DNA sequence was determined by standard techniques known in the art. Oligonucleotides 5901 to 5919 were used, which allow nucleotide sequence determination of both DNA strands. Oligonucleotides 5901 and 5902 hybridize within *nirB* and *htrA*, respectively, while 5919 hybridizes within ptetnir15/mod downstream of the *ureAB* genes. The other oligonucleotides hybridize within the *ureAB* genes. These were diluted to 1 pmol μl^{-1}, packed in dry ice with the plasmid samples, and sent to Cambridge Bioscience (Cambridge) for nucleotide sequence determination. The data confirmed that the nucleotide sequence across the recombinant region of all three plasmids was as expected.

Sequences of primers that can be used in the invention, as is described above, are as follows.

5901
Primes within *nirB* promoter ~60 basepairs upstream of SD sequence
TCA AAT GGT ACC CCT TGC TGA (SEQ ID NO:34)

5902
Primes within *htrA* promoter ~60 basepairs upstream of SD sequence
TAT TCC GGA ACT TCG CGT TA (SEQ ID NO:35)

5903
Primes ~250 basepairs downstream from start of *ureA* gene
TGT TTC CTG ATG GGA CTA AAC TC (SEQ ID NO:36)

5904
Reverse primes ~300 basepairs downstream from start of *ureA* gene
ACC AGG AAC TAA TTT ACC ATT G (SEQ ID NO:37)

5905
Primes ~550 basepairs downstream from start of *ureA* gene
TTG ATT GAC ATT GGC GGT AAC (SEQ ID NO:38)

5906
Reverse primes ~600 basepairs from start of *ureA* gene
GTT GTC TGC TTG TCT ATC AAC C (SEQ ID NO:39)

5907
Primes ~150 basepairs downstream from start of *ureB* gene
GGT GGC GGT AAA ACC CTA AGA G (SEQ ID NO:40)

5908
Reverse primes ~180 basepairs downstream of *ureB* gene
CTT TGC TAG GGT TGT TAG ATT G (SEQ ID NO:41)

5909
Primes ~400 basepairs downstream from start of *ureB* gene
AAT CCC TAC AGC TTT TGC AAG C (SEQ ID NO:42)

5910
Reverse primes ~500 basepairs from start of *ureB* gene
GTG CCA TCA GCA GGA CCG GTT C (SEQ ID NO:43)

5911
Primes ~750 basepairs from start of *ureB* gene
ATC GCC ACA GAC ACT TTG AAT G (SEQ ID NO:44)

5912
Reverse primes ~820 basepairs downstream from start of *ureB* gene
TAG CAG CCA TAG TGT CTT CTA C (SEQ ID NO:45)

5913
Primes ~1050 basepairs downstream from start of *ureB* gene
TGA AGA CAC TTT GCA TGA CAT G (SEQ ID NO:46)

5914
Reverse primes 1080 basepairs downstream of *ureB* gene
TGA GAG TCA GAA CTG GTG ATT G (SEQ ID NO:47)

5915
Primes ~1350 basepairs downstream from start of *ureB* gene
CAT GAT CAT CAA AGG CGG ATT C (SEQ ID NO:48)

5916
Reverse primes ~1380 basepairs downstream from start of *ureB*
GAA GCG TTC GCA TCG CCC ATT TG (SEQ ID NO:49)

5917
Primes ~1650 basepairs from start of *ureB*
TCG TGG ATG GCA AAG AAG TAA C (SEQ ID NO:50)

5918
Reverse primes ~1680 basepairs from start of *ureB*
GCG CCA AGC TCA CTT TAT TG (SEQ ID NO:51)

5919
Reverse primes ~80 basepairs downstream of *Bam*HI site downstream of *ureB*
CAA CGA CAG GAG CAC GAT CAT G (SEQ ID NO:52)

The nucleotide sequences across the promoter regions and *ureAB* genes of the final plasmids, pHUR3 and pNUR3, were also confirmed. *E. coli* MC1061 cells containing the plasmids were sent to Cambridge Biosciences Ltd., who prepared plasmid DNA and determined the nucleotide sequences of the promoter and *ureAB* genes of both plasmids. The data confirmed that the nucleotide sequence across the relevant region of both plasmids was as expected. The sequence of plasmid pHUR3 is

shown in Fig. 4, and a plasmid map showing its relevant features is provided in Fig. 5.

Plasmids pNUR and pHUR were introduced into *S. typhimurium* strains BRD509 and BRD807, and *S. typhi* strains CVD908 and BRD948, by electroporation and selection of ampicillin-resistant colonies.

Immunization and Protection Experiments

Inbred Balb/C mice were immunized by the intragastric route with live, attenuated *Salmonella typhimurium* (1E10 CFU/ml) expressing urease apoenzyme on day 0 (Fig. 1). Animals were boosted twice on days 21 and 35 with 10 μg soluble, recombinant urease plus aluminum hydroxide (200 μg) by the parenteral route. Fourteen days later, serum antibody responses to urease were measured. Controls included: (1) prime-boost with the Salmonella parental control strains (BRD509 ΔaroA/ΔaroD (Strugnell *et al.*, Infection and Immunity 60:3994-4002, 1992) and BRD807ΔaroA/ΔhtrA (Chatfield *et al.*, Microbial Pathogenesis 12:145-151, 1992)) minus the urease construct, (2) mucosal priming with LT in place of Salmonella (gold standard), and (3) parenteral immunization with urease plus alum alone. Attenuated *S. typhimurium* (ΔaroA/ΔaroD) expressing urease under the transcriptional control of either an *htr*A promoter (pHUR3) or the *nir*B promoter (pNUR3) induced an elevated IgG2a response against urease that was greater than the gold standard using LT-Alum (Fig. 2A). A comparable response to LT-Alum was induced with *S. typhimurium* (ΔaroA/ΔhtrA) carrying the same urease constructs (Fig. 2A). Analysis of the IgG1/IgG2a ratio demonstrated the induction of a Th1 response with the double aro mutant, and a more balanced response with the Δaro/ΔhtrA mutant strain (Fig. 2B). Urease-specific antibody in Fig. 2A is expressed as EU/ml on a logarithmic scale and median response is indicated by the bar.

The level of protective efficacy employing *S. typhimurium*-vectored urease in a prime-boost strategy was determined. Fig. 3A shows the results of quantitative *H. pylori* culture of mice immunized on day 0 with 1E10 CFU/ml live attenuated *S. typhimurium* (ΔaroA/ΔaroD or ΔaroA/ΔhtrA) and boosted on days 21 and 35 with urease (10 µg) plus alum (200 µg). Three weeks later, animals were challenged with *H. pylori* (1E7 CFU/ml) and efficacy was assessed in gastric tissue 4 weeks later using quantitative culture. Strains including the urease constructs are indicated in the key of Fig. 3A. Fig. 3B shows protection depicted as $\log_{10}$ reduction in comparison to the no treatment (Tx) control group. A significant reduction in bacterial burden was observed when attenuated Salmonella expressing urease was administered as part of a prime-boost regimen with alum (Wilcoxon rank sum compared to parental control strain). No significant difference was observed between group 1 (pHUR3-Alum) and group 7 (LT-Alum).

All patents and publications cited above are hereby incorporated by reference in their entirety.

What is claimed is:

1. A method of inducing an immune response against Helicobacter in a mammal, said method comprising the steps of:

mucosally administering to said mammal an attenuated Salmonella vector comprising a nucleic acid molecule encoding a Helicobacter antigen, and

parenterally administering to said mammal a Helicobacter antigen.

2. The method of claim 1, wherein said attenuated Salmonella vector is administered orally to said mammal.

3. The method of claim 1, wherein said Helicobacter antigen is a urease, a urease subunit, or an immunogenic fragment thereof

4. The method of claim 1, wherein said mammal is at risk of developing, but does not have, a Helicobacter infection.

5. The method of claim 1, wherein said mammal has a Helicobacter infection.

6. The method of claim 1, wherein said parenteral administration of said Helicobacter antigen further includes parenteral administration of an adjuvant.

7. The method of claim 6, wherein said adjuvant is an aluminum compound.

8. The method of claim 7, wherein said aluminum compound is alum.

9. The method of claim 1, wherein said attenuated Salmonella vector is a *Salmonella typhi* vector.

10. The method of claim 9, wherein said *Salmonella typhi* vector is CVD908-htrA or CVD908.

11. The method of claim 1, wherein the attenuated Salmonella vector is a *Salmonella typhimurium* vector.

12. The method of claim 11, wherein said *Salmonella typhimurium* vector is BRD509 or BRD807.

13. The method of claim 1, wherein said attenuated Salmonella vector further comprises an *htrA* promoter.

14. The method of claim 1, wherein said attenuated Salmonella vector further comprises a *nirB* promoter.

15. The method of claim 1, wherein said mucosal administration primes an immune response to an antigen and said parenteral administration boosts an immune response to said antigen.

16. An attenuated Salmonella vector comprising a nucleic acid molecule encoding a Helicobacter antigen.

17. The vector of claim 16, wherein said antigen is a urease, a urease subunit, or an immunogenic fragment thereof.

18. The vector of claim 16, wherein said attenuated Salmonella vector is a *Salmonella typhi* vector.

19. The vector of claim 18, wherein said *Salmonella typhi* vector is CVD908-htrA or CVD908.

20. The vector of claim 16, wherein the attenuated Salmonella vector is a *Salmonella typhimurium* vector.

21. The vector of claim 20, wherein said *Salmonella typhimurium* vector is BRD509 or BRD807.

22. The vector of claim 16, wherein said attenuated Salmonella vector further comprises an *htrA* promoter.

23. The vector of claim 16, wherein said attenuated Salmonella vector further comprises a *nirB* promoter.

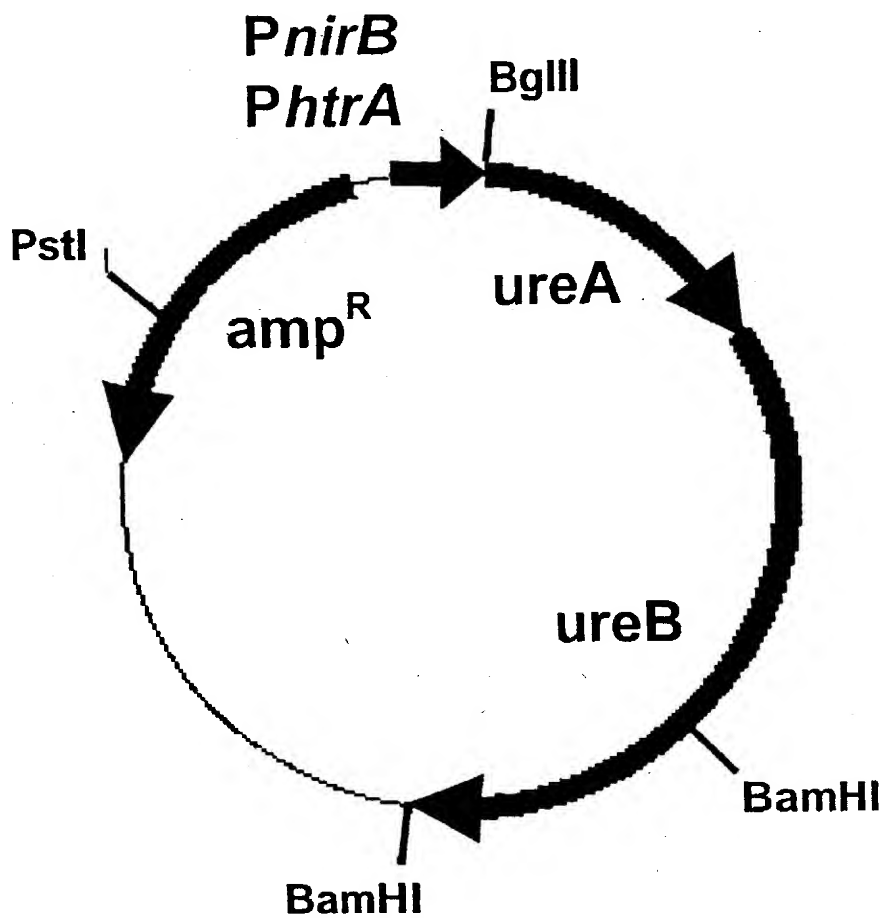


Fig. 1

Figure 2. Mucosal priming with *S. typhimurium* vectored urease followed by parenteral boosting with alum induces an IgG2a humural immune response equivalent to that induced by mucosal priming with LT.

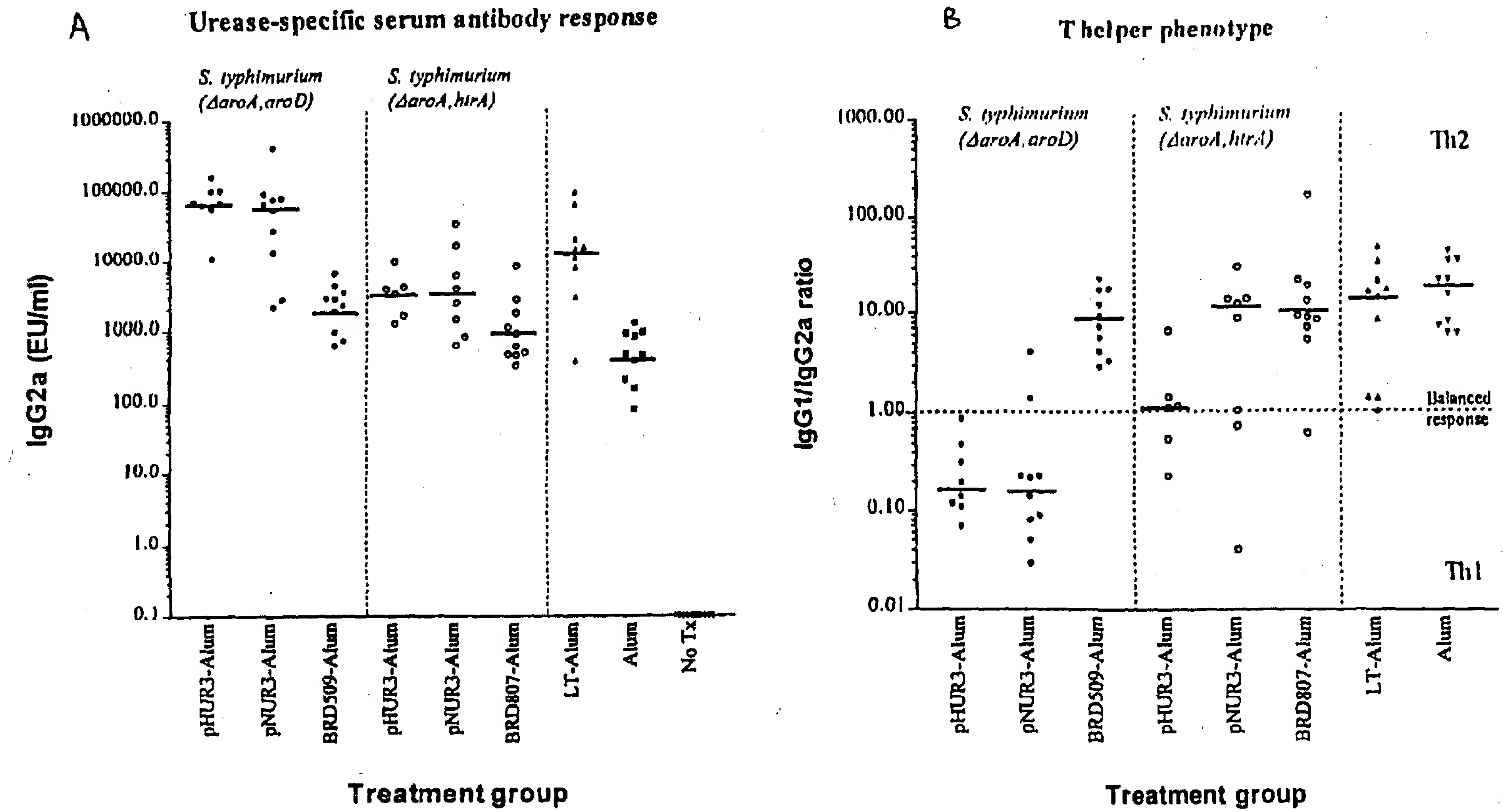
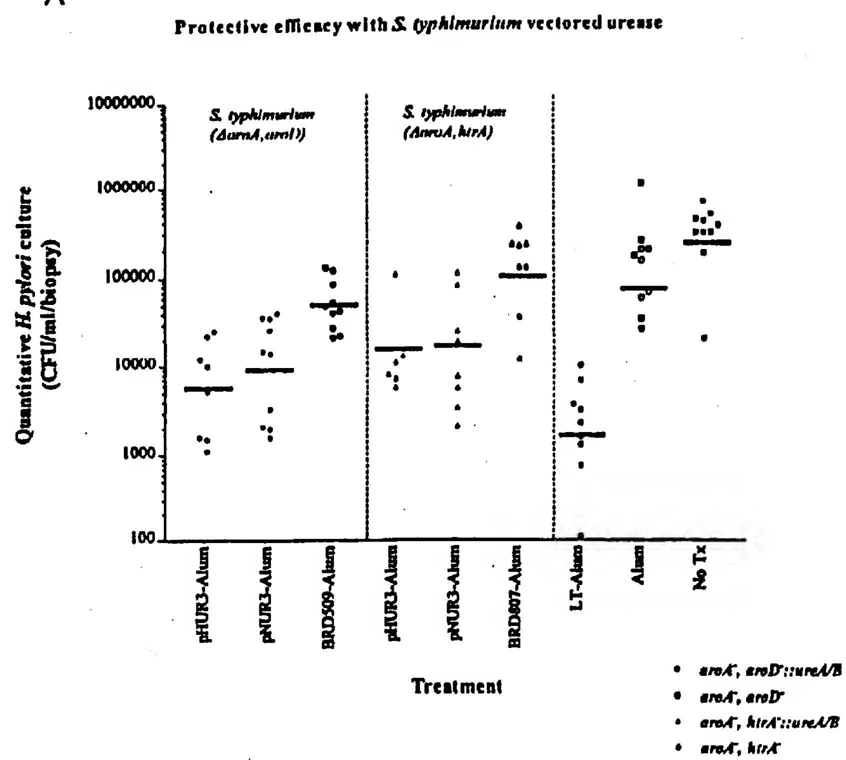


Figure 3. Mucosal priming with attenuated *S. typhimurium::ureA/B* affords equivalent protection as LT in a prime-boost regimen with Alum

A



B

Group	Δ log10 CFU	*P-value*
S. typhimuriumΔaroA,aroD		
1. pHUR3-Alum	1.69	0.0002
2. pNUR3-Alum	1.45	0.003
3. BRD509-Alum	0.75	-
S. typhimuriumΔaroA,htrA		
4. pHUR3-Alum	1.31	0.004
5. pNUR3-Alum	1.29	0.006
6. BRD807-Alum	0.37	-
7. LT-Alum	2.15	<0.0001
8. Alum	0.33	0.01
9. No Tx	-	

Fig. 4 (1 of 4)

EcoR I Bgl II

GAATTCTATTCCGGAACTTCGCGTTATAAAATGAATCTGACGTACACAGCAATTTAGATATTAATCATCCACAGGAGAGATCTCCATGAA 90

PhtrA ▪Ure▪

N S I P E L R V I K . I . R T Q Q F R Y . S S T G E I S M K

ACTCACCCCAAAAGAGTTAGATAAGTTGATGCTCCACTACGCTGGAGAATTGGCTAAAAAACGCAAAGAAAAAGGCATTAAGCTTAACTA 180

UreA

L T P K E L D K L M L H Y A G E L A K K R K E K G I K L N Y

TGTAGAAGCAGTAGCTTTGATTAGTGCCCATATTATGGAAGAAGCGAGAGCTGGTAAAAAGACTGCGGCTGAATTGATGCAAGAAGGGCG 270

UreA

V E A V A L I S A H I M E E A R A G K K T A A E L M Q E G R

CACTCTTTTAAAACCAGATGATGTGATGGATGGCGTGGCAAGCATGATCCATGAAGTGGGTATTGAAGCGATGTTTCCTGATGGGACTAA 360

UreA

T L L K P D D V M D G V A S M I H E V G I E A M F P D G T K

ACTCGTAACCGTGCATACCCCTATTGAGGCCAATGGTAAATTAGTTCCTGGTGAGTTGTTCTTAAAAAATGAAGACATCACTATCAACGA 450

UreA

L V T V H T P I E A N G K L V P G E L F L K N E D I T I N E

AGGCAAAAAAGCCGTTAGCGTGAAAGTTAAAAATGTTGGCGACAGACCGGTTCAAATCGGCTCACACTTCCATTTCTTTGAAGTGAATAG 540

UreA

G K K A V S V K V K N V G D R P V Q I G S H F H F F E V N R

ATGCCTAGACTTTGACAGAGAAAAAACTTTCGGTAAACGCTTAGACATTGCGAGCGGGACAGCGGTAAGATTTGAGCCTGGCGAAGAAAA 630

UreA

C L D F D R E K T F G K R L D I A S G T A V R F E P G E E K

ATCCGTAGAATTGATTGACATTGGCGGTAACAGAAGAATCTTTGGATTTAACGCATTGGTTGATAGACAAGCAGACAACGAAAGCAAAAA 720

UreA

S V E L I D I G G N R R I F G F N A L V D R Q A D N E S K K

AATTGCTTTACACAGAGCTAAAGAGCGTGGTTTTCATGGCGCTAAAAGCGATGACAACTATGTAAAAACAATTAAGGAGTAAGAAATGAA 810

UreA ▪Ure▪

I A L H R A K E R G F H G A K S D D N Y V K T I K E . E M K

AAAGATTAGCAGAAAAGAATATGTTTCTATGTATGGTCCTACTACAGGCGATAAAGTGAGATTGGGCGATACAGACTTGATCGCTGAAGT 900

UreB

K I S R K E Y V S M Y G P T T G D K V R L G D T D L I A E V

AGAACATGACTACACCATTTATGGCGAAGAGCTTAAATTCGGTGGCGGTAAAACCCTAAGAGAAGGCATGAGCCAATCTAACAACCCTAG 990

UreB

E H D Y T I Y G E E L K F G G G K T L R E G M S Q S N N P S

CAAAGAAGAGTTGGATTTAATTATCACTAACGCTTTAATCGTGGATTACACCGGTATTTATAAAGCGGATATTGGTATTAAAGATGGCAA 1080

UreB

K E E L D L I I T N A L I V D Y T G I Y K A D I G I K D G K

AATCGCTGGCATTGGTAAAGGCGGTAACAAAGACATGCAAGATGGCGTTAAAAACAATCTTAGCGTAGGTCCTGCTACTGAAGCCTTAGC 1170

UreB

I A G I G K G G N K D M Q D G V K N N L S V G P A T E A L A

CGGTGAAGGTTTGATCGTAACGGCTGGTGGTATTGACACACACATCCACTTCATTTCACCCCAACAAATCCCTACAGCTTTTGCAAGCGG 1260

UreB

G E G L I V T A G G I D T H I H F I S P Q Q I P T A F A S G

Fig. 4 (2 of 4)

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TGTAACAACCATGATTGGTGGTGGAACCGGTCCTGCTGATGGCACTAATGCGACTACTATCACTCCAGGCAGAAGAAATTTAAAATGGAT 1350
UreB
 V  T  T  M  I  G  G  G  T  G  P  A  D  G  T  N  A  T  T  I  T  P  G  R  R  N  L  K  W  M
GCTCAGAGCGGCTGAAGAATATTCTATGAATTTAGGTTTCTTGGCTAAAGGTAACGCTTCTAACGATGCGAGCTTAGCCGATCAAATTGA 1440
UreB
 L  R  A  A  E  E  Y  S  M  N  L  G  F  L  A  K  G  N  A  S  N  D  A  S  L  A  D  Q  I  E
AGCCGGTGCGATTGGCTTTGCAATTCACGAAGACTGGGGCACCACTCCTTCTGCAATCAATCATGCGTTAGATGTTGCGGACAAATACGA 1530
UreB
 A  G  A  I  G  F  A  I  H  E  D  W  G  T  T  P  S  A  I  N  H  A  L  D  V  A  D  K  Y  D
TGTGCAAGTCGCTATCGCCACAGACACTTTGAATGAAGCCGGTTGTGTAGAAGACACTATGGCTGCTATTGCTGGACGCACTATGCACAC 1620
UreB
 V  Q  V  A  I  A  T  D  T  L  N  E  A  G  C  V  E  D  T  M  A  A  I  A  G  R  T  M  H  T
TTTCCACACTGAAGGCGCTGGCGGCGGACACGCTCCTGATATTATTAAAGTAGCCGGTGAACACAACATTCTTCCCGCTTCCACTAACCC 1710
UreB
 F  H  T  E  G  A  G  G  G  H  A  P  D  I  I  K  V  A  G  E  H  N  I  L  P  A  S  T  N  P
CACCATCCCTTTCACCGTGAATACAGAAGCAGAGCACATGGACATGCTTATGGTGTGCCACCACTTGGATAAAAGCATTAAAGAAGATGT 1800
UreB
 T  I  P  F  T  V  N  T  E  A  E  H  M  D  M  L  M  V  C  H  H  L  D  K  S  I  K  E  D  V
                Bam HI
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UreB
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                              Bam HI
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Fig. 4 (3 of 4)

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Origin

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Origin

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Origin

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Origin

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Origin

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AmpR

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AmpR

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AmpR

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AmpR

A E V V L Q L Y P P P S S L L I V A G K L E . V V R Q L I V

Fig. 4 (4 of 4)

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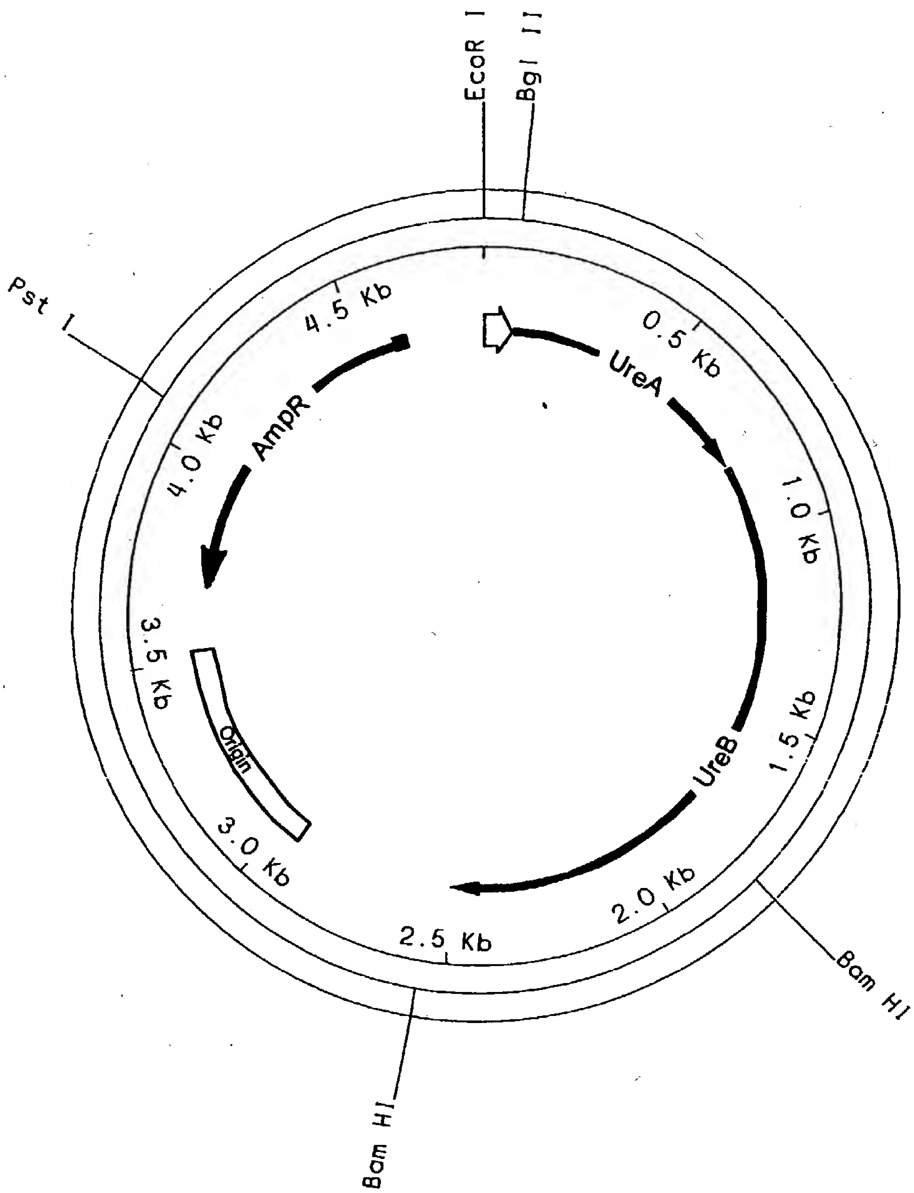
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Fig. 5

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cca aaa gag tta gat aag ttg atg ctc cac tac gct gga gaa ttg gct   145
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Asn Thr Glu Ala Glu His Met Asp Met Leu Met Val Cys His His Leu
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1035 1040 1045

ctt cgg gaa gcg tgg cgc ttt ctc aat gct cac gct gta ggt atc tca 3217
Leu Arg Glu Ala Trp Arg Phe Leu Asn Ala His Ala Val Gly Ile Ser
1050 1055 1060

gtt cgg tgt agg tcg ttc gct cca agc tgg gct gtg tgc acg aac ccc 3265
Val Arg Cys Arg Ser Phe Ala Pro Ser Trp Ala Val Cys Thr Asn Pro
1065 1070 1075 1080

ccg ttc agc ccg acc gct gcg cct tat ccg gta act atc gtc ttg agt 3313
Pro Phe Ser Pro Thr Ala Ala Pro Tyr Pro Val Thr Ile Val Leu Ser
1085 1090 1095

cca acc cgg taa gac acg act tat cgc cac tgg cag cag cca ctg gta 3361
Pro Thr Arg Asp Thr Thr Tyr Arg His Trp Gln Gln Pro Leu Val

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            1100                1105                1110

aca gga tta gca gag cga ggt atg tag gcg gtg cta cag agt tct     3406
Thr Gly Leu Ala Glu Arg Gly Met     Ala Val Leu Gln Ser Ser
            1115                    1120                1125

tga agt ggt ggc cta act acg gct aca cta gaa gga cag tat ttg gta     3454
    Ser Gly Gly Leu Thr Thr Ala Thr Leu Glu Gly Gln Tyr Leu Val
                    1130                1135                1140

tct gcg ctc tgc tga agc cag tta cct tcg gaa aaa gag ttg gta gct     3502
Ser Ala Leu Cys     Ser Gln Leu Pro Ser Glu Lys Glu Leu Val Ala
                    1145                1150                1155

ctt gat ccg gca aac aaa cca ccg ctg gta gcg gtg gtt ttt ttg ttt     3550
Leu Asp Pro Ala Asn Lys Pro Pro Leu Val Ala Val Val Phe Leu Phe
                1160                1165                1170

gca agc agc aga tta cgc gca gaa aaa aag gat ctc aag aag atc ctt     3598
Ala Ser Ser Arg Leu Arg Ala Glu Lys Lys Asp Leu Lys Lys Ile Leu
            1175                1180                1185

tga tct ttt cta cgg ggt ctg acg ctc agt gga acg aaa act cac gtt     3646
    Ser Phe Leu Arg Gly Leu Thr Leu Ser Gly Thr Lys Thr His Val
            1190                1195                1200

aag gga ttt tgg tca tga gat tat caa aaa gga tct tca cct aga tcc     3694
Lys Gly Phe Trp Ser     Asp Tyr Gln Lys Gly Ser Ser Pro Arg Ser
        1205                1210                1215

ttt taa att aaa aat gaa gtt tta aat caa tct aaa gta tat atg agt     3742
Phe     Ile Lys Asn Glu Val Leu Asn Gln Ser Lys Val Tyr Met Ser
            1220                1225                1230

aaa ctt ggt ctg aca gtt acc aat gct taa tca gtg agg cac cta tct     3790
Lys Leu Gly Leu Thr Val Thr Asn Ala     Ser Val Arg His Leu Ser
        1235                1240                1245

cag cga tct gtc tat ttc gtt cat cca tag ttg cct gac tcc ccg tcg     3838
Gln Arg Ser Val Tyr Phe Val His Pro     Leu Pro Asp Ser Pro Ser
        1250                1255                1260

tgt aga taa cta cga tac ggg agg gct tac cat ctg gcc cca gtg ctg     3886
Cys Arg     Leu Arg Tyr Gly Arg Ala Tyr His Leu Ala Pro Val Leu
            1265                1270                1275

caa tgataccgcg agacccacgc tcaccggctc cagatttatc agcaataaac     3939
Gln

cagccagccg gaagggccga gcgcagaagt ggtcctgcaa ctttatccgc ctccatccag 3999
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<212> PRT
<213> Salmonella typhimurium

<400> 2
Asn Ser Ile Pro Glu Leu Arg Val Ile Lys
1 5 10

<210> 3
<211> 7
<212> PRT
<213> Salmonella typhimurium

<400> 3
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1 5

<210> 4
<211> 245
<212> PRT
<213> Artificial Sequence

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Helicobacter pylori.

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Lys Leu Met Leu His Tyr Ala Gly Glu Leu Ala Lys Lys Arg Lys Glu
20 25 30
Lys Gly Ile Lys Leu Asn Tyr Val Glu Ala Val Ala Leu Ile Ser Ala
35 40 45

His Ile Met Glu Glu Ala Arg Ala Gly Lys Lys Thr Ala Ala Glu Leu
50 55 60
Met Gln Glu Gly Arg Thr Leu Leu Lys Pro Asp Asp Val Met Asp Gly
65 70 75 80
Val Ala Ser Met Ile His Glu Val Gly Ile Glu Ala Met Phe Pro Asp
85 90 95
Gly Thr Lys Leu Val Thr Val His Thr Pro Ile Glu Ala Asn Gly Lys
100 105 110
Leu Val Pro Gly Glu Leu Phe Leu Lys Asn Glu Asp Ile Thr Ile Asn
115 120 125
Glu Gly Lys Lys Ala Val Ser Val Lys Val Lys Asn Val Gly Asp Arg
130 135 140
Pro Val Gln Ile Gly Ser His Phe His Phe Phe Glu Val Asn Arg Cys
145 150 155 160
Leu Asp Phe Asp Arg Glu Lys Thr Phe Gly Lys Arg Leu Asp Ile Ala
165 170 175
Ser Gly Thr Ala Val Arg Phe Glu Pro Gly Glu Glu Lys Ser Val Glu
180 185 190
Leu Ile Asp Ile Gly Gly Asn Arg Arg Ile Phe Gly Phe Asn Ala Leu
195 200 205
Val Asp Arg Gln Ala Asp Asn Glu Ser Lys Lys Ile Ala Leu His Arg
210 215 220
Ala Lys Glu Arg Gly Phe His Gly Ala Lys Ser Asp Asp Asn Tyr Val
225 230 235 240
Lys Thr Ile Lys Glu
245

<210> 5
<211> 570
<212> PRT
<213> Helicobacter pylori

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Glu Met Lys Lys Ile Ser Arg Lys Glu Tyr Val Ser Met Tyr Gly Pro
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Val Glu His Asp Tyr Thr Ile Tyr Gly Glu Glu Leu Lys Phe Gly Gly
35 40 45
Gly Lys Thr Leu Arg Glu Gly Met Ser Gln Ser Asn Asn Pro Ser Lys
50 55 60
Glu Glu Leu Asp Leu Ile Ile Thr Asn Ala Leu Ile Val Asp Tyr Thr
65 70 75 80
Gly Ile Tyr Lys Ala Asp Ile Gly Ile Lys Asp Gly Lys Ile Ala Gly
85 90 95
Ile Gly Lys Gly Gly Asn Lys Asp Met Gln Asp Gly Val Lys Asn Asn
100 105 110
Leu Ser Val Gly Pro Ala Thr Glu Ala Leu Ala Gly Glu Gly Leu Ile
115 120 125
Val Thr Ala Gly Gly Ile Asp Thr His Ile His Phe Ile Ser Pro Gln

130 135 140
Gln Ile Pro Thr Ala Phe Ala Ser Gly Val Thr Thr Met Ile Gly Gly
145 150 155 160
Gly Thr Gly Pro Ala Asp Gly Thr Asn Ala Thr Thr Ile Thr Pro Gly
165 170 175
Arg Arg Asn Leu Lys Trp Met Leu Arg Ala Ala Glu Glu Tyr Ser Met
180 185 190
Asn Leu Gly Phe Leu Ala Lys Gly Asn Ala Ser Asn Asp Ala Ser Leu
195 200 205
Ala Asp Gln Ile Glu Ala Gly Ala Ile Gly Phe Ala Ile His Glu Asp
210 215 220
Trp Gly Thr Thr Pro Ser Ala Ile Asn His Ala Leu Asp Val Ala Asp
225 230 235 240
Lys Tyr Asp Val Gln Val Ala Ile Ala Thr Asp Thr Leu Asn Glu Ala
245 250 255
Gly Cys Val Glu Asp Thr Met Ala Ala Ile Ala Gly Arg Thr Met His
260 265 270
Thr Phe His Thr Glu Gly Ala Gly Gly Gly His Ala Pro Asp Ile Ile
275 280 285
Lys Val Ala Gly Glu His Asn Ile Leu Pro Ala Ser Thr Asn Pro Thr
290 295 300
Ile Pro Phe Thr Val Asn Thr Glu Ala Glu His Met Asp Met Leu Met
305 310 315 320
Val Cys His His Leu Asp Lys Ser Ile Lys Glu Asp Val Gln Phe Ala
325 330 335
Asp Ser Arg Ile Arg Pro Gln Thr Ile Ala Ala Glu Asp Thr Leu His
340 345 350
Asp Met Gly Ile Phe Ser Ile Thr Ser Ser Asp Ser Gln Ala Met Gly
355 360 365
Arg Val Gly Glu Val Ile Thr Arg Thr Trp Gln Thr Ala Asp Lys Asn
370 375 380
Lys Lys Glu Phe Gly Arg Leu Lys Glu Glu Lys Gly Asp Asn Asp Asn
385 390 395 400
Phe Arg Ile Lys Arg Tyr Leu Ser Lys Tyr Thr Ile Asn Pro Ala Ile
405 410 415
Ala His Gly Ile Ser Glu Tyr Val Gly Ser Val Glu Val Gly Lys Val
420 425 430
Ala Asp Leu Val Leu Trp Ser Pro Ala Phe Phe Gly Val Lys Pro Asn
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Met Ile Ile Lys Gly Gly Phe Ile Ala Leu Ser Gln Met Gly Asp Ala
450 455 460
Asn Ala Ser Ile Pro Thr Pro Gln Pro Val Tyr Tyr Arg Glu Met Phe
465 470 475 480
Ala His His Gly Lys Ala Lys Tyr Asp Ala Asn Ile Thr Phe Val Ser
485 490 495
Gln Ala Ala Tyr Asp Lys Gly Ile Lys Glu Glu Leu Gly Leu Glu Arg
500 505 510
Gln Val Leu Pro Val Lys Asn Cys Arg Asn Ile Thr Lys Lys Asp Met
515 520 525
Gln Phe Asn Asp Thr Thr Ala His Ile Glu Val Asn Pro Glu Thr Tyr
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His Val Phe Val Asp Gly Lys Glu Val Thr Ser Lys Pro Ala Asn Lys
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Val Ser Leu Ala Gln Leu Phe Ser Ile Phe
565 570

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<212> PRT
<213> Salmonella typhimurium

<400> 6
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20 25 30
Leu Ala Thr Gly Ala His Asp Arg Ala Pro Val Val Glu Asp Pro Ala
35 40 45
Arg Leu Ala Gly Leu Pro Tyr Trp Leu Ala Glu
50 55

<210> 7
<211> 67
<212> PRT
<213> Salmonella typhimurium

<400> 7
Ile Thr Asp Thr Arg Ala Asn Val Lys Arg Leu Leu Leu Gln Asn Val
1 5 10 15
Cys Asp Leu Ser Asn Asn Met Asn Gly Leu Arg Phe Pro Cys Phe Val
20 25 30
Lys Ser Gly Asn Ala Glu Val Ser Ala Leu Pro Leu Pro Arg Ser Leu
35 40 45
Thr Arg Cys Ala Arg Ser Phe Gly Cys Gly Glu Arg Tyr Gln Leu Thr
50 55 60
Gln Arg Arg
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<211> 141
<212> PRT
<213> Salmonella typhimurium

<400> 8
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Gln Lys Ala Ser Lys Arg Pro Gly Thr Val Lys Arg Pro Arg Cys Trp
20 25 30
Arg Phe Ser Ile Gly Ser Ala Pro Leu Thr Ser Ile Thr Lys Ile Asp

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Ala Gln Val Arg Gly Gly Glu Thr Arg Gln Asp Tyr Lys Asp Thr Arg
    50                  55                  60
Arg Phe Pro Leu Glu Ala Pro Ser Cys Ala Leu Leu Phe Arg Pro Cys
65                  70                  75                  80
Arg Leu Pro Asp Thr Cys Pro Pro Phe Ser Leu Arg Glu Ala Trp Arg
                85                  90                  95
Phe Leu Asn Ala His Ala Val Gly Ile Ser Val Arg Cys Arg Ser Phe
            100                 105                 110
Ala Pro Ser Trp Ala Val Cys Thr Asn Pro Pro Phe Ser Pro Thr Ala
        115                 120                 125
Ala Pro Tyr Pro Val Thr Ile Val Leu Ser Pro Thr Arg
    130                 135                 140
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<210> 9
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<212> PRT
<213> Salmonella typhimurium

<400> 9

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Glu Arg Gly Met
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<210> 10
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<213> Salmonella typhimurium

<400> 10

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Ala Val Leu Gln Ser Ser
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<210> 11
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<212> PRT
<213> Salmonella typhimurium

<400> 11

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Ala Leu Cys
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<210> 12
<211> 43

<212> PRT
<213> Salmonella typhimurium

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20 25 30
Arg Ala Glu Lys Lys Asp Leu Lys Lys Ile Leu
35 40

<210> 13
<211> 20
<212> PRT
<213> Salmonella typhimurium

<400> 13
Ser Phe Leu Arg Gly Leu Thr Leu Ser Gly Thr Lys Thr His Val Lys
1 5 10 15
Gly Phe Trp Ser
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<210> 14
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<212> PRT
<213> Salmonella typhimurium

<400> 14
Asp Tyr Gln Lys Gly Ser Ser Pro Arg Ser Phe
1 5 10

<210> 15
<211> 23
<212> PRT
<213> Salmonella typhimurium

<400> 15
Ile Lys Asn Glu Val Leu Asn Gln Ser Lys Val Tyr Met Ser Lys Leu
1 5 10 15
Gly Leu Thr Val Thr Asn Ala
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<210> 16
<211> 15
<212> PRT
<213> Salmonella typhimurium

<400> 16
Ser Val Arg His Leu Ser Gln Arg Ser Val Tyr Phe Val His Pro
1 5 10 15

<210> 17
<211> 8
<212> PRT
<213> Salmonella typhimurium

<400> 17
Leu Pro Asp Ser Pro Ser Cys Arg
1 5

<210> 18
<211> 14
<212> PRT
<213> Escherichia coli

<400> 18
Leu Arg Tyr Gly Arg Ala Tyr His Leu Ala Pro Val Leu Gln
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<210> 19
<211> 4824
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tagataacta cgatacggga gggcttacca tctggcccca gtgctgcaat ga tac cgc 3898
                                                          Tyr Arg
                                                           1

gag acc cac gct cac cgg ctc cag att tat cag caa taa acc agc cag  3946
Glu Thr His Ala His Arg Leu Gln Ile Tyr Gln Gln     Thr Ser Gln
        5                   10                      15

ccg gaa ggg ccg agc gca gaa gtg gtc ctg caa ctt tat ccg cct cca  3994
Pro Glu Gly Pro Ser Ala Glu Val Val Leu Gln Leu Tyr Pro Pro Pro
        20                  25                  30

tcc agt cta tta att gtt gcc ggg aag cta gag taa gta gtt cgc cag  4042
Ser Ser Leu Leu Ile Val Ala Gly Lys Leu Glu     Val Val Arg Gln
    35                  40                      45

tta ata gtt tgc gca acg ttg ttg cca ttg ctg cag gca tcg tgg tgt  4090
Leu Ile Val Cys Ala Thr Leu Leu Pro Leu Leu Gln Ala Ser Trp Cys
    50                  55                  60

cac gct cgt cgt ttg gta tgg ctt cat tca gct ccg gtt ccc aac gat  4138
His Ala Arg Arg Leu Val Trp Leu His Ser Ala Pro Val Pro Asn Asp
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65                  70                  75                  80

caa ggc gag tta cat gat ccc cca tgt tgt gca aaa aag cgg tta gct   4186
Gln Gly Glu Leu His Asp Pro Pro Cys Cys Ala Lys Lys Arg Leu Ala
                85                  90                  95

cct tcg gtc ctc cga tcg ttg tca gaa gta agt tgg ccg cag tgt tat   4234
Pro Ser Val Leu Arg Ser Leu Ser Glu Val Ser Trp Pro Gln Cys Tyr
            100                 105                 110

cac tca tgg tta tgg cag cac tgc ata att ctc tta ctg tca tgc cat   4282
His Ser Trp Leu Trp Gln His Cys Ile Ile Leu Leu Leu Ser Cys His
        115                 120                 125

ccg taa gat gct ttt ctg tga ctg gtg agt act caa cca agt cat tct   4330
Pro     Asp Ala Phe Leu     Leu Val Ser Thr Gln Pro Ser His Ser
        130                 135                 140

gag aat agt gta tgc ggc gac cga gtt gct ctt gcc cgg cgt caa cac   4378
Glu Asn Ser Val Cys Gly Asp Arg Val Ala Leu Ala Arg Arg Gln His
        145                 150                 155

ggg ata ata ccg cgc cac ata gca gaa ctt taa aag tgc tca tca ttg   4426
Gly Ile Ile Pro Arg His Ile Ala Glu Leu     Lys Cys Ser Ser Leu
    160                 165                     170

gaa aac gtt ctt cgg ggc gaa aac tct caa gga tct tac cgc tgt       4471
Glu Asn Val Leu Arg Gly Glu Asn Ser Gln Gly Ser Tyr Arg Cys
    175                 180                 185

tga gat cca gtt cga tgt aac cca ctc gtg cac cca act gat ctt cag   4519
    Asp Pro Val Arg Cys Asn Pro Leu Val His Pro Thr Asp Leu Gln
        190                 195                 200

cat ctt tta ctt tca cca gcg ttt ctg ggt gag caa aaa cag gaa ggc   4567
His Leu Leu Leu Ser Pro Ala Phe Leu Gly Glu Gln Lys Gln Glu Gly
    205                 210                 215

aaa atg ccg caa aaa agg gaa taa ggg cga cac gga aat gtt gaa tac   4615
Lys Met Pro Gln Lys Arg Glu     Gly Arg His Gly Asn Val Glu Tyr
220                 225                     230

tca tac tct tcc ttt ttc aat att att gaa gca ttt atc agg gtt att   4663
Ser Tyr Ser Ser Phe Phe Asn Ile Ile Glu Ala Phe Ile Arg Val Ile
235                 240                 245                 250

gtc tca tga gcg gat aca tat ttg aat gta ttt aga aaa ata aac aaa   4711
Val Ser     Ala Asp Thr Tyr Leu Asn Val Phe Arg Lys Ile Asn Lys
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Lys Arg Leu Ala Pro Ser Val Leu Arg Ser Leu Ser Glu Val Ser Trp
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